PROTOPLASMIC DIFFERENCES BETWEEN MESOPHILES AND THERMOPHILES[1]

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The biologist is plagued by the immense difficulty involved in the discovery of general laws by which all living organisms abide. He cannot possibly examine all forms of life, but dare he generalize before he has done so? The second quarter of this century has provided him with the comforting point of view of "comparative physiology," which is based on the premise that unity existing among organisms may be discernible from the study of "obviously" different forms of life. This intellectual shortcut narrowed the distance between him and his goal. However, unity is in the eyes of the beholder! One observer may be awed by the recognition that different types of organisms seem to read the same metabolic maps. Others are struck by the fact that even organisms that appear to follow similar metabolic patterns may show recognizable differences in other respects. Obviously then superimposed upon the observed unity are mechanisms by which diversity can be achieved.

Several possibilities exist for the expression of biological individuality on the molecular level. First, organisms or species may be distinct entities because of differences in their metabolic machinery that allow them to perform unique chemical reactions. Some possess the know-how to manufacture unusual materials that give them distinct characteristics, while others have never acquired this ability or lost it in the course of evolution. Many are capable of utilizing alternative pathways in their metabolism, a versatility that permits them a degree of adaptability unknown to organisms that enjoy fewer biochemical opportunities. However, individualization need not depend solely on *qualitative* differences, but also can be brought about by *quantitative* and *organizational* differences. We still have the tendency of regarding metabolic patterns as two-dimensional abstractions of a three-dimensional reality. Though the metabolic pathways existing in one organism may appear the same on the printed page as those occurring in another organism, they may differ in their arrangement in space, the load of molecular traffic that they can handle, the quantity of traffic they need to accommodate under given situations, and the policing system employed. Furthermore, even identical molecules or cellular structures can be organized in space in many ways, each arrangement contributing a mark of distinction. It is the last problem, namely architectural individualization, that I should like to exemplify with our experience on the nature of biological thermostability.

The problem. The existence of life at temperatures at and sometimes above 70 C (3, 21, 26) has been known for almost two hundred years. The most widely distributed and studied forms have been microorganisms. The problem is: how do these organisms manage to live at temperatures at which ordinarily heat-sensitive cellular constituents are destroyed?

At least two general types of explanations for this remarkable faculty have been offered (3, 21, 26). The most evident is that the essential cell components of thermophiles are relatively more stable than those of mesophiles. This stability may be an inherent property of these molecules and molecular complexes (*i.e.*, *intrinsic* or *true* stability), or may be brought about through the presence of protective factors, or the absence of labilizing materials, in the external or internal environment of the cell (*i.e.*, *stabilization* or *apparent* stability). A second school of thought, as represented by the writings of Gaughran (21) and of Allen (3), believes that rapid resynthesis of damaged or destroyed cell constituents is the key to the problem of biological stability to heat.

[1] Eli Lilly and Company Award address in Bacteriology and Immunology. Presented at the Fifty-seventh Annual Meeting of the Society of American Bacteriologists in Detroit, Michigan, on May 1, 1957.

[2] The Rockefeller Foundation and the Office of Naval Research supported the initial phases of this work; since 1954 we have received a grant from the U. S. Public Health Service. I am also indebted to former and present associates who shared in this work.

Concerning the first hypothesis, many of the indispensable materials of "normal" cells are fairly heat-labile. Certain lipids, proteins, and complexes of these compounds with each other and other types of molecules are the most obvious examples. It is an interesting fact that animals and plants that live at lower temperatures have fats with lower melting points than those living at higher temperatures. Heilbrunn (25) and later Bělehrádek (7, 8) pointed out that differences in the sensitivity of various organisms toward heat can be correlated with the differences in the melting points of their lipids. Thermal death may be due to the solution of certain lipids. This is an intriguing possibility, especially because some organisms accustomed to relatively cold environments have been reported to die of "heat" at astonishingly low temperatures, in one case as low as 11.5 C (26). On cursory examination it is difficult to imagine that death under these conditions results from protein denaturation by heat directly. However, such an assumption is unsafe because alterations of normal enzymes so as to make them unexpectedly heat-labile have been shown to be inducible by mutation (28, 40). Organisms having lived in a cold environment for generations may have experienced mutations that caused some of their critical proteins or protein complexes to become relatively heat-labile.[3] Such mutations need not express themselves as lethal ones unless these organisms are transferred to a warmer environment. For example, the protoplasm of sea urchin eggs coagulates when these cells are held at 34 C. On the basis of circumstantial evidence Heilbrunn believes that this coagulation does not represent the direct effect of heat, but of calcium, which is released from the cortex of heated cells, together with lipid from a protein-lipid-calcium complex (26, 27). As attractive as the "lipid-solubilization" theory is, it still requires decisive experimental evidence. Its promise as a working hypothesis, however, is indicated by Gaughran's preliminary finding (22) that the lipid content of a mesophile growing at temperatures above its optimum decreases as the temperature is increased, and that the lipid becomes more saturated; the lipids of a thermophile, on the other hand, remain relatively constant in quantity and degree of saturation. Furthermore, one of Militzer's students, Dyer, found that the phospholipids of the thermophile *Bacillus stearothermophilus* 2184 consist almost entirely of sphingomyelins, which have a higher melting point than the more customary phospholipids (18). These important leads ought to be followed up. H. M. Kagan and I recently studied the heat stability of cytoplasmic membranes isolated from *Bacillus megaterium* by the method of Weibull (84), and found them to be astonishingly heat-stable. The optical density of membrane suspensions remained unchanged during and after heating at 100 C for 30 min, as did the appearance of

TABLE 1

*Coagulability of cytoplasmic proteins from mesophilic and thermophilic bacteria**

Cytoplasmic Proteins	Protein in Solution†		Protein Coagulated
	Time of heating (min)		
	0	8	8
	mg/ml	*mg/ml*	%
Proteus vulgaris	2.9	1.3	55
Escherichia coli	3.1	1.4	55
Bacillus megaterium	2.4	1.0	58
Bacillus subtillis	2.8	1.2	57
Bacillus stearothermophilus NCA 2184	3.3	3.2	3
Bacillus sp. Purdue CD	2.9	2.9	0
Bacillus sp. Texas 11330	2.8	2.7	4
Bacillus sp. Nebraska 1492	2.8	2.8	0

* At pH 6, 60 C, for 8 min.

† Determined as trichloroacetic acid precipitable material.

TABLE 2

*Effect of protein concentration on the coagulability of cytoplasmic proteins**

Proteus vulgaris		*Bacillus stearothermophilus*	
Conc protein at 0 time	Protein coagulated	Conc protein at 0 time	Protein coagulated
mg/ml	%	*mg/ml*	%
10.1	92	10.0	11
5.9	78	6.1	8
3.1	54	3.2	3
0.6	25	0.6	0

* At pH 6, 60 C, for 8 min.

[3] Cookbooks contain many clues concerning the heat-stability of various proteins. Fish and seafood need to be cooked gently.

TABLE 3

*Effect of pH on the coagulability of cytoplasmic proteins**

pH	Protein Coagulated	
	Proteus vulgaris	*Bacillus stearothermophilus*
	%	%
5	91	7
6	56	4
7	50	4
8	42	0
9	22	0

* At 60 C, for 8 min.

these cellular structures as examined with the electron microscope. Admittedly, more subtle alterations could have occurred that escaped detection.

Evidence for the relative heat stability of proteins from thermophiles is fairly substantial. For example, various enzymes from thermophiles have been found to be unusually heat-stable (12, 13, 24, 29, 46, 47, 49, 50–52, 54, 57–59, 67). In some cases it has been demonstrated that the thermal resistance of such enzymes is dependent upon the temperature at which the organisms from which the enzymes were isolated had been grown (11, 72). This information, to which Militzer, Georgi, and their associates have made especially valuable contributions, suggests that at least certain proteins of thermophiles appear to be more heat-stable than corresponding molecules from mesophilic cells. Unfortunately, the available data, except Campbell's (12, 13), were obtained with impure enzymes, and thus do not enable one to distinguish between inherent molecular stability and external stabilization. Campbell's experiments, however, were performed with purified enzymes. He showed that a crystalline amylase isolated from cultures of *Bacillus coagulans* grown at 55 C is markedly more heat-stable than that isolated from cultures grown at 35 C. Though one could argue that even crystalline proteins may contain stabilizing contaminants (60, 66, 71) in minute yet effective concentrations, the most reasonable explanation of Campbell's finding is that the observed difference in relative thermostability is due to molecular differences and not to external substances. Campbell's observation confirmed our own conclusions to be discussed later regarding the thermostability of flagella from thermophiles.

The crude model. Another question left unanswered was whether the observed relative heat-resistance (regardless of its basis) is the property of only a few enzymes or is characteristic for the bulk of cytoplasmic proteins. Gale and I showed (table 1) that cytoplasmic proteins isolated from thermophiles (last 4 organisms) are strikingly more stable than similar preparations from mesophiles (32, 43). The crude mixtures of cytoplasmic proteins used constituted approximately half of the total cell nitrogen. Over 50 per cent of the mesophile proteins, but hardly any of the thermophile proteins, coagulate when they are heated at 60 C and pH 6 for 8 min. The extent of coagulation is affected by the initial protein concentration (table 2) and pH (table 3). In every case, though, are the thermophile proteins more heat-stable than the mesophile proteins. Since deoxyribonucleic acid (23) and cell particles (68) have been reported to stabilize certain proteins, these materials were removed from the preparations, but without significant effect on the thermostability of thermophile proteins. Furthermore, a mixture of proteins from a mesophile and a thermophile when heated behaves as one would expect on the basis of the relative heat stabilities of the individual preparations (table 4). This suggests that no stabilizing or labilizing factors were released from either preparation during heating. Though this does not exclude the possibility that stabilization of heat-labile molecules plays a role in the survival of thermophiles, the intrinsic stability of these components seems to be a more likely basis for biological stability to heat. Thermophile proteins are heat-

TABLE 4

Lack of interaction concerning coagulability between preparations of mesophile and thermophile proteins

Cytoplasmic Proteins	Protein in Solution	
	Time of heating (min)	
	0	8
	mg/ml	*mg/ml*
Proteus vulgaris (1)	0.21	0.16
Bacillus stearothermophilus (2)	0.21	0.21
(1) + (2) calculated	0.42	0.37
(1) + (2) observed	0.42	0.37

* At pH 6, 60 C.

TABLE 5

Antibody-binding and antigenic properties of flagella and flagellin

Reaction		Maximum Antibody N Ppt'd	Antibody N/Antigen N
Antigen	Antibodies		
		mg	
Flagella	Antiflagella	0.48	1.1
Flagellin (A)	Antiflagella	0.064-0.080	6.3–7.4
Flagellin (H)	Antiflagella	0.052–0.086	5.7–6.3
Flagellin (A)	Antiflagellin (A)	0.40	12.7
Flagellin (H)	Antiflagellin (H)	0.41–0.48	10.7–15.1
Flagella	Antiflagellin (H)	0.51	0.9

Flagellin (A) = Flagellin obtained when flagella were dissociated at pH 2.5 and 30 C for 10 min; the pH of the resulting solution was adjusted to 7.

Antiflagellin (A) = Antibodies against flagellin (A).

Flagellin (H) = Flagellin obtained when flagella were dissociated at 60 C for 30 min.

Antiflagellin (H) = Antibodies against flagellin (H).

stable in cell free condition and in an aqueous environment; therefore theories explaining the extraordinary properties of thermophiles on the basis of protective cell coatings or the unavailability of moisture (3, 21, 26) are not convincing.

Since our experiments can be criticized on the basis that the conditions used are not representative of normal cellular organization, and that heating may have caused more subtle alterations than coagulation, we decided to continue working with a more suitable test system. This was especially necessary, because the nature of the observed stability cannot be studied critically with a mixture of substances. Fortunately, by that time as a by-product of our interest in bacterial motility (36, 37, 45, 63–65) we had an excellent test material for this study at our disposal.

The refined model. Locomotion in most of the true bacteria is probably brought about by flagella (4, 69, 74, 81).[4] As examined in the electron microscope, flagella appear as wavy structures, which are flattened during the drying of the preparations; in reality flagella are helical. Relatively little was known regarding their chemistry (10, 41) until the Swedish worker Weibull examined the properties of flagella from *Proteus vulgaris* and *Bacillus subtilis*, and found them to consist of protein (75, 76, 79, 82). Flagella apparently belong to a large class of structural proteins, the keratin-myosin-epidermin-fibrinogen (k-m-e-f) group, as can be deduced from X-ray diffraction patterns (4).

Flagella can be isolated by fractional centrifugation alone (14, 17, 20, 75, 76, 79), or by a combination of centrifugation and precipitation with ammonium sulfate (33, 34, 62, 76). In either case, the cells, after thorough washing, are first shaken vigorously to remove the flagella (6, 14, 15, 20, 45, 55, 70, 75, 85, 86). The cell bodies and the debris then are removed by centrifugation at 3400 × G (1 hr, 3×), and the flagella are centrifuged and washed at 35,000 × G (2 hr, 2×). Purified flagellar preparations are clear, and impurities usually are apparent by their colored or opaque appearance. Such impurities, if present, are almost always found in the center-bottom portion of the precipitate, and in our experience one of the most successful purification steps consists of removing these centers by suction or by cutting off the bottom of the tube. Fractional precipitation with ammonium sulfate can be substituted for this (33, 34, 62, 76).

Isolated flagella appear like those still attached to cells, except that smaller pieces are present, probably as a result of breakage during the shaking procedure. Flagellar suspensions are viscous, show a marked Tyndall effect and birefrigence of flow (75, 76, 79). Flagella disintegrate when held at a pH below 3 to 4 (17, 33–35, 62,

[4] For a more recent statement of another point of view see the paper by Pijper which appeared while this manuscript was in press (Bacterial flagella and motility. Ergeb. Mikrobiol. Immunitätsforsch. u. Exptl. Therap., **30**, 37–91, 1957)

75, 76, 79, 85); the typical morphology, viscosity, and the other properties disappear under these conditions. In an intensive study on the chemistry of flagella from 3 mesophilic and 3 thermophilic species, Kobayashi and I used pH 2 at room temperature for 30 min as a standard condition, and found that all preparations tested disintegrate when treated in this matter. Heat (1, 2, 9, 16, 19, 38, 43, 44, 48, 53, 56), compounds capable of breaking hydrogen bonds (38, 42–44), and the detergent sodium dodecylsulfate (SDS) (38, 44) also bring about the disintegration of flagella. In the few instances that they studied, Weibull and Tiselius (85), and later Weibull (75, 76, 79), found that the products of acid disintegration are relatively uniform in size, as judged by ultracentrifugation. These products probably represent the protein molecules, recently named "flagellins" (4), of which flagella are constructed, or small multiples thereof. From ultracentrifugation data Weibull concluded that the flagellin from *P. vulgaris* consisted of elongated molecules that had a molecular weight of *ca.* 40,000. This is approximately twice the value that we obtained for the flagellin from another strain of this organism by other means (as will be mentioned later), and that S. Erlander, J. F. Foster, and I recently determined from the sedimentation characteristics of that molecule. In work to be described elsewhere we found that under certain conditions flagellin dimerizes and polymerizes; it is therefore likely that the value obtained by Weibull relates to the dimer rather than the monomer of flagellin.

Apparently, then, flagella are highly organized aggregates of flagellin molecules held together by bonds that are fairly easily broken. Whether the flagellin prepared by the dissociation of flagella has the same properties as the molecules in the flagellum is as yet unknown. Though flagellin can be reaggregated in many orderly patterns, nobody has yet succeeded in rebuilding flagella from flagellin. This may be an indication that the molecules have been altered during the dissociation of the flagellum, but it could also be an expression of the fact that the flagellum is a complex structure. Some immunochemical data (61) obtained with the quantitative precipitin method may have some bearing on this question (table 5). Flagellin solutions prepared by relatively mild acid or heat treatment of flagella react only with approximately 15 per cent of the antibodies directed against highly purified flagella. Yet, flagella react quantitatively almost the same as do flagellin molecules with antibodies against flagellin molecules. This suggests that the flagellum possesses certain antibody-binding sites that are no longer present or accessible in the free flagellin molecules, while the flagellum seems to contain all such sites present in the flagellin molecules. Among possible interpretations are that the flagellin molecules bound together in the flagellum have properties that individual molecules do not have, or that they become denatured by the method of preparation, as the organization of the flagellum becomes destroyed.

Impure preparations of flagella, after disintegration at pH 2, yield a precipitate (referred to in

TABLE 6

*Chemical properties of highly purified flagella**

Flagella From	Fraction [Saturation with $(NH)_2SO_4$]	Per Cent						
		Yield	N	"pH 2 insol. materials"	Anthrone-positive materials	Pentose	Nucleic acids	Ash
Proteus vulgaris	10–20	15.6	16.6	<0.01	<0.001	<0.001	0.01	0.01
Serratia marcescens	20–50	35.5	15.8	<0.01	<0.01	0.06	0.05	—
Bacillus subtilis	20–35	34.7	16.7	<0.003	<0.006	<0.0002	<0.0006	<0.003
Bacillus sp. NCA 2184	20–35	31.2	16.2	<0.01	<0.002	<0.001	<0.002	0.005
Bacillus sp. Purdue CD	20–35	21.5	16.2	<0.01	0.2	<0.001	—	—
Bacillus sp. Texas 11330	30–40	14.6	15.7	<0.01	<0.01	<0.05	0.10	—

* Relatively impure suspensions of flagella at 2 to 4 C were fractionated with ammonium sulfate at 10 per cent intervals. This table gives the composition of the fractions showing the highest purity. Preparations obtained by fractional centrifugation (plus removal of the center-bottom portion of the precipitate, as is indicated in the text) are of similar purity.

this discussion as "pH 2 insoluble material"), when they are centrifuged at the relative centrifugal forces by which normal flagella can be made to settle out. This precipitate contains the bulk of nonprotein substances (70 to 84 per cent of the anthrone-positive material contained in the preparation, 67 to 88 per cent of the pentose, 88 to 90 per cent of the nucleic acid, and 85 to 100 per cent of the ash). Consequently, the amount of residue after treatment of flagella at pH 2 is one of the best criteria for the presence of contaminants (33–35, 75, 76, 79). Preparations that are considered purified by other criteria almost always are free from "pH 2 insoluble materials" (34, 79).

As shown by Weibull (77, 80), and before him by Craigie (14), and Gard (20), flagella can be precipitated with ammonium sulfate. Under such conditions flagella aggregate into bundles and larger orderly patterns. While some flagella can be precipitated at all concentrations of ammonium sulfate tried, the nonflagellar contaminants apparently can be precipitated more selectively. In general, the 0 to 10 and the 50 to 100 per cent fractions are the least pure, and by discarding them one can isolate fairly purified preparations of flagella. To obtain material of the highest purity additional fractionation is needed (34). Table 6 shows the properties of the type of material that can be isolated routinely and without great difficulty.

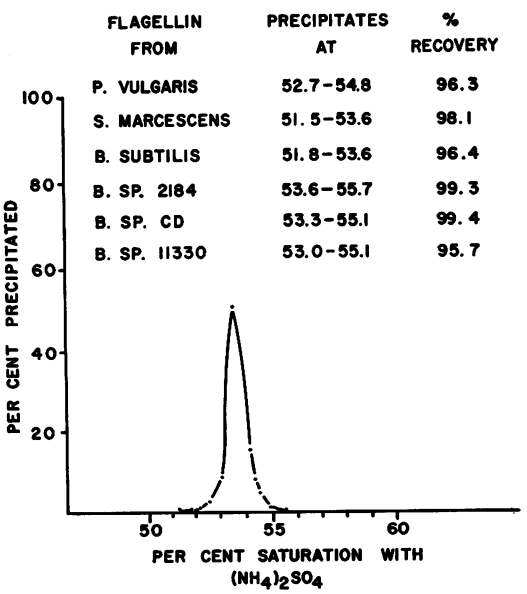

Figure 1. Precipitation of flagellin with ammonium sulfate.

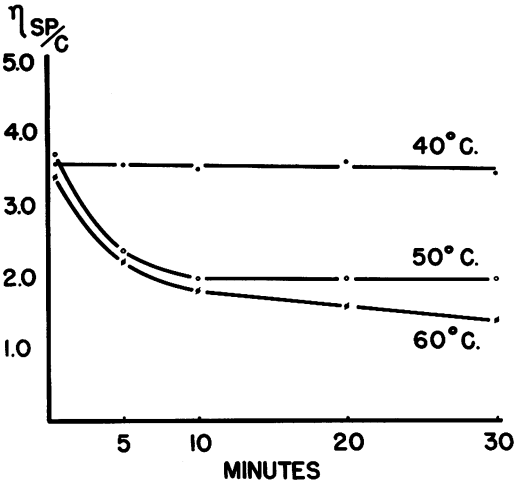

Figure 2. Effect of various temperatures on the reduced viscosity of suspensions of flagella from the mesophile *Escherichia coli*.

A purified protein should contain only one N-terminal amino acid, unless it consists of more than one peptide chain or a branched peptide chain. Applying the Sanger method to various ammonium sulfate fractions of the 6 preparations of flagella shown in table 6, and chromatographically separating the dinitrophenyl derivatives, we found that highly purified preparations never contain any other accessible amino groups than the α group of alanine and the ϵ group of lysine (35, 83). Less purified preparations contain small amounts of other N-terminal amino acids.

The reason why some flagella can be precipitated at all the concentrations of ammonium sulfate used seems to lie in the fact that the preparations contain flagella of various lengths and in different stages of aggregation. This interpretation is supported by the observation that when the morphological features of flagella are destroyed by mild acid treatment, the liberated flagellin molecules can be precipitated within a very narrow range of ammonium sulfate concentrations (figure 1). The uniformity of the peak obtained as well as the absence of other peaks is another indication that the preparation is homogeneous.

Kobayashi and I recently crystallized flagellin (from *P. vulgaris*) by the addition of ethanol at pH 3.0 to 3.5 to a final concentration of 60 per cent. The crystals obtained contained 15.8 per

cent nitrogen, and gave gray to light-gray interference colors when examined in a polarizing microscope and distinct X-ray diffraction patterns.

Weibull reported that the flagella of *P. vulgaris* and *B. subtilis* do not contain any cysteine or cystine (76, 82). This does not agree with our observations (33, 35, 62). Even our best preparations contain from 0.54 to 0.75 per cent cysteine or cystine. The presence of one or both of these compounds is not likely due to contaminants, because such impurities would have to be present in large concentrations to account for the level of cysteine or cystine found. Furthermore, if one calculates the minimal molecular weight of flagellin molecules on the basis that each molecule contains 1 molecule of cysteine one arrives at values (16,000 to 22,500 depending upon the species from which the flagellin was obtained) similar to those obtained on the basis that each flagellin molecule contains 1 N-terminal alanine (13,900 to 20,800). We cannot distinguish between cysteine and cystine by the method of analysis employed. Therefore, if one bases the calculations of the minimal molecular weights on the assumption that each flagellin molecule contains 1 molecule of cystine (rather than cysteine) twice the indicated values would be obtained. As mentioned previously, our recent sedimentation data indicate, at least for flagellin molecules from *P. vulgaris*, that a molecular weight in the neighborhood of 20,000 is most likely.

In spite of the fact that flagellin molecules are architecturally similar to the proteins of the k-m-e-f class, in their amino acid composition they differ from all of them (5, 31, 73, 79). The molecular formula of flagellin from *P. vulgaris* based on seven determinations and a molecular weight of 17,300 is as follows (62): $Asp_{27}Glu_{16}(Amide)_{23}Arg_6His_0Lys_8Cys/2_1Tyr_2HOPro_0Met_1Ser_{11}Thr_{14}Gly_{14}Ala_{18}Val_{10}Leu_{14}Ileu_9Pro_0Phe_4Try_0$. Striking are the absence of histidine, hydroxyproline, proline, and tryptophane, the low level of sulfur-containing amino acids, a relatively high concentration of threonine, and the fact that aspartic acid occurs in larger amounts than glutamic acid, a relationship not reported for other proteins (82). Neither purified whole flagella nor acid prepared flagellin molecules are digested by proteolytic enzymes like trypsin, pepsin, or papain (33, 62).

Towards understanding the molecular basis of thermostability. I hope I have convinced you by now that flagella are an excellent test system for a study of relative heat-stability. First, no other bacterial protein can be isolated as easily in purified form by the same method from many different organisms. Secondly, flagella are highly organized complexes of protein molecules which may simulate in general style intracellular structural materials, about which we know practically nothing; in any case, they lend themselves to a study of intermolecular stability. Thirdly, since the dissociation of flagella into flagellin molecules is accompanied by a decrease of viscosity, the stability of flagellar suspensions can be determined by viscosity measurements. For example, figure 2 shows the effect of temperature on suspensions of flagella from *Escherichia coli* over a period of 30 min. Exposure to 40 C has no effect, but 50 C or more causes the disintegration of the flagella. The flagella of a thermophile, on the other hand (figure 3) are much more stable. Disintegration in this case begins at 75 C. The data in table 7 prepared from curves like these show the remaining specific viscosity after exposure of the flagella to various temperatures for 20 min. Disintegration of flagella from mesophiles becomes marked at temperatures higher than 50 C, while flagella from thermophiles remain intact at temperatures as high as 70 C. This is in agreement with the experience of McCoy (48), who showed that the H-agglutinogen of *Clostridium thermosaccharo-*

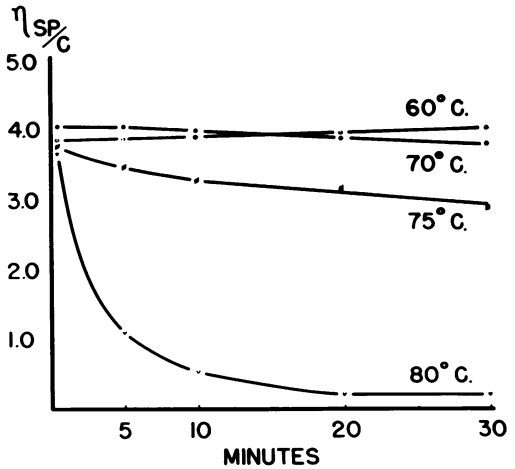

Figure 3. Effect of various temperatures on the reduced viscosity of suspensions of flagella from the thermophile *Bacillus* sp. 11330.

TABLE 7

Difference in heat stability between flagella from mesophiles and thermophiles

Flagella From	Per Cent Initial Specific Viscosity after 20 min at C:						
	40	50	60	65	70	75	80
Proteus vulgaris	99	67	18	—	—	—	—
Escherichia coli	100	56	36	—	—	—	—
Bacillus megaterium	100	72	28	—	—	—	—
Bacillus subtilis	—	98	79	48	31	—	—
Bacillus stearothermophilus NCA 2184	—	—	—	100	—	43	18
Bacillus sp. Purdue CD	—	—	—	100	99	94	41
Bacillus sp. Texas 11330	100	—	105	—	96	79	5
Bacillus sp. Nebraska 39	100	—	112	—	94	56	14

lyticum, a thermophile, is much more heat-stable than that of *C. butyricum*, a mesophile. In general, the heat stability of the flagella used corresponds to the ability of the organisms from which they were isolated to grow at high temperatures. For example, the strain of *Bacillus subtilis* used, for which the maximum growth temperature is between that of the mesophiles and that of the thermophiles, produces flagella that are intermediate in heat-stability.

The question as to whether the relative heat-stability of thermophile flagella is an inherent characteristic or is provided by protective materials (3, 21, 23, 60, 66, 68, 71) was considered in a number of experiments (1, 2, 38). Though our inability to demonstrate the existence of protective materials does not prove the absence of such substances, we came to the tentative conclusion that the relative stability of the flagellum is due to true intermolecular stability. We then tried to find out more about the nature of that stability, by comparing the behavior of thermostable and thermolabile flagella in the presence of various protein denaturing agents.

If the relative heat-stability of thermophile flagella is due to more abundant or more strategically located disulfide linkages, they should be more resistant to thioglycolate, an agent known to break disulfide bridges. Similarly, if the relative stability of thermophile flagella is brought about by more abundant or stronger hydrogen bonding, urea, acetamide, and other agents thought to break hydrogen bonds should be less damaging to thermophile than to mesophile flagella. Thirdly, if bonds between the hydrophobic residues (alanine, valine, leucine, isoleucine and phenylalanine) play a significant stabilizing role, then detergents like SDS, which by some protein chemists is thought to disrupt the so-called hydrophobic bonds (30), should depolymerize mesophile flagella more readily than thermophile flagella.

The possibility that thermophile flagella are more stable because of more effective disulfide bonding does not seem likely (38). First, flagella contain only small amounts of cysteine-cystine, regardless of whether they have been produced by mesophiles or thermophiles; each molecule of flagellin contains probably one molecule of cysteine or cystine. Secondly, flagella dissociate under relatively mild conditions, such as below pH 3 to 4. Thirdly, thioglycolate in a concentration known to denature other proteins does not affect the stability of flagella, though it should be granted that in the native flagellum the disulfide bridges may not be accessible. In any case, these bonds do not seem to be major stabilizing factors in the architecture of either mesophile or thermophile flagella.

On the other hand, there seem to exist striking differences in hydrogen bonding (38, 42–44). For example, thermophile flagella are more resistant to urea than are mesophile flagella (table 8). The mesophile flagella are rapidly disintegrated by 6 M urea; the thermophile flagella are not affected by that concentration, but will dissociate when the concentration of urea is raised to 9 M. Flagella from thermophiles are also more stable to acetamide than are flagella from mesophilic bacteria. Eight to 10 M acetamide destroys mesophile flagella, but has no detectable effect on thermophile flagella. Inasmuch as urea and acetamide

are regarded as compounds capable of breaking hydrogen bonds, it seems likely that more "effective" hydrogen bonding is involved in the relative thermostability of flagella from thermophiles. The flagella of *B. subtilis*, which are intermediate in heat-stability, are also intermediate in their stability to urea. Since we were unable, even after diligent search, to demonstrate any stabilizing factors in thermophile flagella (or labilizing materials in mesophile flagella), we concluded that the stability to urea, like heat stability, probably represents an intrinsic property (38, 42–44).

Trying to analyze the role of salt linkages in the stability of flagella, we prepared titration curves for mesophile and thermophile flagella, like the one shown in figure 4. The titration was performed (78) first from pH 6 to pH 10.5 (solid line), then from 10.5 to 2.5 (broken line). Below pH 3 to 4 the flagellum dissociates to flagellin, and on back-titration to pH 10.5 (dotted line) the solution behaves as if additional titratable groups have become available. In the intact flagellum such groups may not be titratable because they are sterically inaccessible, or combined in linkages. However, the difference in titration curves also may be due to changes in the acid or basic nature of the ionizable groups after the flagellum has broken apart. Contrary to our expectations we did not find a correlation between the relative heat-stability of the flagella and the apparent increase in the number of titratable groups after dissociation of the flagella by acid; however, we observed a striking difference in the total number of H^+ ions bound at pH 2.5 and 10.5 (38, 44). This is shown in table 9. The flagellin molecules isolated from thermophilic bacteria apparently possess only about half as many basic and acidic groups as do flagellin molecules from mesophiles. This finding suggests another explanation for the relative stability of the former. Flagellin molecules in the flagellum may face sites carrying charges of like sign. Because of the resulting electrostatic repulsion such molecules would have a tendency to dissociate. The main difference between mesophile and thermophile flagella therefore may not reside in differences in binding, but in a greater or lesser tendency of the constituent flagellin molecules to repel each other. The titration data also can be interpreted somewhat differently (38). In any case, though, it is clear that the amphoteric properties of mesophile and thermophile flagella differ remarkably, and that these differences probably constitute useful clues regarding the architecture of bacterial flagella.

Thermophile flagella apparently are more resistant to the action of SDS than are mesophile flagella (table 8). The concentration of SDS has to be increased to two and one-half times the amount used in this experiment before thermophile flagella begin to dissociate (38, 44). The mechanism by which anionic detergents denature proteins is not completely understood, but it is thought that they first react with basic groups. The greater resistance of thermophile flagella to the action of SDS, therefore, may be merely an expression of the fact that they possess fewer

TABLE 8

Difference between flagella from mesophiles and thermophiles in stability to urea, acetamide, and sodium dodecylsulfate (SDS)

Flagella From	Per Cent Initial Reduced Viscosity in Presence of:					
	6 M urea				10 M acetamide	0.0035 M SDS
	After minutes:					
	5	10	30	60	30	60
Proteus vulgaris	4	9	1	1	11	67
Escherichia coli	16	9	7	7	—	—
Bacillus megaterium	23	17	8	7	17	60
Bacillus subtilis	83	72	43	21	13	63

Flagella from the following thermophilic strains did not show any decrease in viscosity: *Bacillus stearothermophilus* NCA 2184, *Bacillus* sp. Purdue CD, Texas 11330, and Nebraska 39.

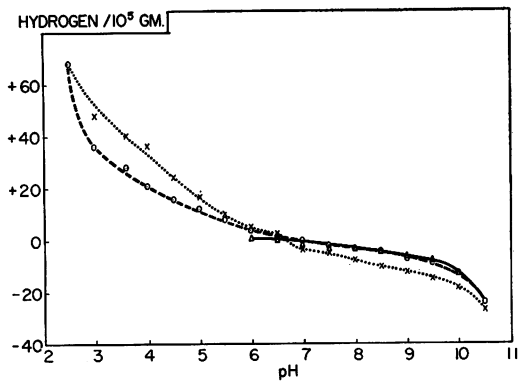

Figure 4. Titration curves for flagella (solid and broken lines) and flagellin (dotted line) from *Bacillus* sp. 11330.

accessible basic groups to which the SDS can be anchored than do mesophile flagella. However, the amount of SDS necessary to cause the observed disintegration of flagella is in excess of that theoretically needed to neutralize the available basic groups, and the dissociation of flagellin molecules in reality may come about through the breakage of hydrophobic bonds by the detergent (38, 44). Hydrophobic bonding is thought to be caused by the tendency of relatively nonpolar amino acid residues to avoid water and to adhere to each other (30). Such bonding may play an important role in holding the flagellin molecules together in the flagellum. The nonpolar portion of the SDS is more likely to stick to the nonpolar residues of the flagellin than to enter the aqueous phase, and this may result in the rupture of existing hydrophobic bonds. The relatively greater resistance of thermophile flagella to SDS, therefore, may be due to stronger, more numerous, or more critically located hydrophobic bonds than occur in mesophile flagella. However, this situation needs to be studied further.

In addition to the explanations mentioned, one has to consider the possibility that the stability of thermophile flagella is provided by novel types of bonding. No information regarding this has been discovered so far.

In spite of numerous chemical, physical, and morphological similarities, in their heat stability flagella from thermophilic bacteria differ strikingly from mesophile flagella. Since flagella are organized structures composed of fibrous proteins, this relative stability is a reflection of the forces holding these molecules together. The relative stability of flagella appears to be an intrinsic property of these structures, since no stabilizing or labilizing materials could be demonstrated. Thermophile flagella are also more stable than mesophile flagella to urea and acetamide, agents regarded as hydrogen bond breakers, and to SDS, an anionic detergent. These findings are interpreted to imply that the relative stability of thermophile flagella is due to more numerous, stronger, or more critically located hydrogen and/or possible hydrophobic bonds than are present in mesophile flagella. The latter seem to contain twice the number of titratable groups as do the former. This suggests that the repulsion between charged groups having like signs may result in a less stable configuration of the flagellin complex.

Summing up: We have shown that the cytoplasmic proteins of thermophiles are markedly more stable than comparable preparations from mesophiles. Using flagella (which are polymers of fibrous proteins) as models, we have found clues that might explain the remarkable heat stability of thermophile proteins. These studies, though still rudimentary, illustrate the molecular basis of biological differences.

At the onset of this text I mentioned several hypotheses that might account for life at high temperatures, but I have not yet taken a position regarding the possibility that this phenomenon is made possible by the rapid repair of damaged molecules or cellular structures (3, 21).

Death by heat has been postulated to be brought about through the injury of a few essential molecules, perhaps only one. Within limits, inactivation of a cell is reversible as long as there

TABLE 9

Differences in H^+-binding capacity between flagellins from mesophiles and thermophiles

Flagellin From	Minimum Molecular Weight	H^+ Bound/Mole	
		pH 2.5	pH 10.5
Proteus vulgaris	19,600	+24	−12
Serratia marcescens	20,800	+23	−11
Bacillus subtilis	18,200	+19	−9
Bacillus stearothermophilus NCA 2184	13,900	+8	−8
Bacillus sp. Purdue CD	15,200	+7	−5
Bacillus sp. Texas 11330	16,700	+10	−4

is the opportunity for repair. Conversely, when such an opportunity is lacking the loss of viability may become irreversible. Yet, in certain systems striking differences in susceptibility to heat can be exhibited, even when resynthesis is not possible. For example, *E. coli* phage T5 gives rise to mutants that are about 5000 times more heat resistant than is the parent strain (39). Since the heat inactivation of these particles takes place in the absence of host cells, *i.e.*, when synthetic activities are not being performed, the lesser heat susceptibility of the mutant strains is most likely due to greater architectural stability of the particles.

Furthermore, if rapid synthesis really is the key to the problem of life at high temperatures, why can't mesophiles grow at 70 C? Rates of reactions double or triple with every 10 C rise in temperature. Theoretically then, an organism should be from 16 to 81 times as active at 70 C as it is at 30 C, as long as denaturation of essential molecules does not become the dominant reaction. Unless it can be proved that thermophiles are capable of performing biochemical reactions at 70 C more rapidly than can be anticipated on that basis, there is no need to assume that they are remarkable in that respect. For repairs to be made the shop, the tools, and the blueprints for items to be repaired must be in usable condition. I am inclined to think that the uniqueness of true thermophiles lies in the relative stability of their critical molecules or structures.

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